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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Prostate cancer is the second highest cause of cancer death in Western society men. Early stages are treatable, but late stage disease is incurable with palliative care the only option.

Purpose: We will use a combination of gene therapy approaches to target prostate cancer locally and in other organs (metastases). Gene directed enzyme/pro-drug therapy (GDEPT) is delivered in the prostate. This kills cancer cells and generates an immune reaction. To augment this immune response, and reduce metastatic tumor growth, locally delivered cytokine gene therapy, is injected with the GDEPT.

Scope: A fusion gene derived from *E. coli* (cytosine deaminase and uracil phosphoribosyltransferase) will be delivered by a lentivirus vector into the prostate of tumor-bearing mice that are then treated with the clinically approved pro-drug, 5 fluorocytosine (5FC). Cytokine genes, delivered in a plasmid, will be injected to complement the GDEPT. These cytokines can augment the maturation, proliferation and anti-tumor cytotoxic activity of cellular components of the immune system.

Results/Progress: Due to difficulties in obtaining the original materials, we have obtained a no-cost extension, and using new materials, work will commence in 2003.

Significance: These model systems will provide valuable preclinical data to support a phase I clinical trial.

14. SUBJECT TERMS:
preclinical studies, gene therapy, GDEPT, cytokine gene therapy

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TITLE: Formulated Delivery of Enzyme/Pro-Drug and Cytokine Gene Therapy to Promote Immune Reduction of Treated and Remote Tumors in Mouse Models of Prostate Cancer

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Formulated delivery of enzyme/pro-drug and cytokine gene therapy to promote immune reduction of treated and remote tumors in mouse models of prostate cancer

Annual Report, DAMD17-02-1-0107

INTRODUCTION:

The mortality and morbidity of prostate cancer (CaP), the most common cancer in men in Western society, occur as a result of late stage disease. Whilst early stages of prostate cancer can be treated in various ways, late stage disease is currently incurable, and palliative care is the only option. The **subject** of this work is to compare new therapies by combining different gene therapies to treat late stage prostate cancer. When cancer cells are killed, an immune response is generated against them. We hypothesize that this immune response can be augmented by administering cytokine genes locally at the same time as gene therapy that kills cancer cells. Our **purpose** is to compare the use of gene-directed enzyme pro-drug therapy (GDEPT) and cytokine gene therapy either alone or in combination in their ability to reduce the size of tumors grown in the prostate and pseudo-metastases induced in the lung by intravenous administration. The **scope** of research involves preparing lentivirus vectors containing the genes for GDEPT (a fusion gene derived from E.coli comprising cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT)). Mice with prostate tumors will be injected into the prostate with the vector and subsequently treated with the clinically approved pro-drug, 5 fluorocytosine (5FC). CD-GDEPT with 5FC has been used safely in a clinical trial for colon cancer. CD/UPRT converts 5FC to the freely diffusible metabolite, 5-fluorouracil (5FU) and two other products, that can enter the metabolic pathways for both DNA and RNA. Thus, *both dividing and non-dividing cells can be killed, which is of benefit because prostate tumors are characterized by a low proportion of dividing cells.* We are establishing a collaboration with Dr Paul Rennie, Vancouver, to use a prostate-directed promoter based on elements of the probasin promoter to control the CD/UPRT gene so that gene expression and cell killing is limited as far as possible to the target cells. To date, relatively little work has been done using the CD/UPRT-GDEPT system. Similarly, although certain cytokine genes are being extensively investigated, we will investigate the ability of two candidates, IL-12 and IL-18 delivered in a plasmid (pCytokine) to complement targeted cell killing. These cytokines can augment the maturation, proliferation and anti-tumor cytotoxic activity of natural killer and T cells and moreover, have been found in other studies to be synergistic. We will investigate the nature of the immune response generated and the effects of the treatments either alone or in combination in inhibiting prostate cancer growth in the prostate and in metastatic sites. This work will provide preclinical data to allow the work to proceed to clinical trial in patients with prostate cancer.

BODY:

We were not able to commence the work as it was originally outlined in our application. This application was submitted by a consortium of researchers, Professor Pamela J Russell from University of New South Wales, and Drs Gerry Both, Peter Molloy and Trevor Lockett from Commonwealth Scientific and Industrial Research Organisation (CSIRO). CSIRO, that was to supply the materials for the project, does not wish to continue the studies. The University of New South Wales has therefore applied for and obtained a no-cost extension based on a new statement of work that uses commercially available materials and virus vectors rather than the proprietory materials from CSIRO. This extension was to begin on 1st January 003.

KEY RESEARCH ACCOMPLISHMENTS:

Nil at this time.

REPORTABLE OUTCOMES:

There are no reportable outcomes.

CONCLUSIONS:

There are no conclusions at this time.

A copy of the New Statement of Work follows. PLEASE NOTE THAT ALL CHANGES TO ORIGINAL ARE IN BOLD

Formulated Delivery of Enzyme/Pro-Drug and Cytokine Gene Therapy to Promote Immune Reduction of Treated and Remote Tumors in Mouse Models of Prostate Cancer

Principal Investigator: Pamela J Russell, PhD

Technical Abstract

Background: Prostate cancer is now the most common cancer and the second highest cause of cancer death in men in Western society. While early stage disease can be treated in several ways, late stage disease is presently incurable and often only palliative care is available.

Objective: Our objective is to use a combination of gene therapy approaches to target both local prostate cancer and its metastases.

Supporting rationale: Gene therapy offers new possibilities for treatment. Of the several strategies available we are investigating the use of *gene directed enzyme/pro-drug therapy (GDEPT) accompanied by the use of locally delivered lipid and cytokine gene therapy*, alone and in combination. The concept is that local delivery of a cell-killing agent, coupled with help from the immune system can effect a reduction in tumor size both at the treatment site and at remote tumor locations i.e. a 'distant bystander' effect is induced. We have chosen to use a **fusion gene** derived from *E. coli*, **comprising cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT), in combination with the clinically approved pro-drug, 5 fluorocytosine (5FC),** as the cell killing system. CD-GDEPT with 5FC has been used safely in a clinical trial for colon cancer. **CD/UPRT converts 5FC to the freely diffusible metabolite, 5-fluorouracil (5FU)** and two other products, that can enter the metabolic pathways for both DNA and RNA. Thus, *both dividing and non-dividing cells can be killed, which is of benefit because prostate tumors are characterized by a low proportion of dividing cells.* **We are establishing a collaboration with Dr Paul Rennie, Vancouver, to use a prostate-directed promoter based on elements of the probasin promoter** to control the CD/UPRT gene so that gene expression and cell killing is limited as far as possible to the target cells. To date, relatively little work has been done using the CD/UPRT-GDEPT system. Similarly, although certain cytokine genes are being extensively investigated, we will investigate the ability of two candidates, IL-12 and **IL-18** delivered in a plasmid (pCytokine) to complement targeted cell killing. These cytokines can augment the maturation, proliferation and anti-tumor cytotoxic activity of natural killer and T cells and moreover, **have been found in other studies to be synergistic**. A key factor in our work is how efficiently the therapeutic genes can be delivered to target tissues. We will use a **lentivirus to deliver the GDEPT** gene cassette. **Lentiviruses have been shown to provide gene expression over an extended time, can infect non-dividing as well as dividing cells and are not subject to immune responses that inhibit human adenovirus delivery systems**. This may provide a delivery advantage in the clinic. The cytokine gene of choice, controlled by the **human/rodent ferritin promotor and chimpanzee/rodent elongation factor 1 alpha (EF1α) promoter** (commercially available) will be delivered in a plasmid, permitting its dose to be adjusted independently of the viral dose.

Significance: If we are successful in reducing tumor volume and metastases in these model systems the work will provide valuable preclinical data to support a phase I clinical trial. In the clinic, our approach of combination treatment will require patients with an accessible tumor who have a significant likelihood of disseminated disease, e.g. localised disease recurrence after prostatectomy or radiation therapy. If proven effective the approach would be applicable both to patients with existing metastatic disease and to those with localised disease, but with a high risk of progression following definitive local treatment. Treatment could be applied prior to a radical prostatectomy *to promote a systemic immune response while the disseminated tumor burden is low.*

Specific Aims: To assess the ability of **lenti-viral delivery of a novel CD/UPRT-GDEPT virus,** pCytokine or a combination of both to suppress orthotopic and metastatic prostate cancer in the RM-1 and TRAMP models. We also aim to show that lentivirus containing a gene cassette driven by prostate directed regulatory regions can effect gene expression in various human prostate tissue slices by fluorescence microscopy.

Study Design: We will use two immunocompetent mouse models. In the first murine RM-1 prostate cancer cells are implanted orthotopically to promote local tumor growth, or given intravenously to establish metastatic tumors in the lungs. In the second, TRAMP mice will be used. These transgenic mice reflect the progression of prostate cancer as it occurs in man. The GDEPT/ lipid-formulated cytokine agents are then injected directly into prostate tumors, either separately, or in combination. We will monitor the extent and repertoire of immune cells that infiltrate the tumor in response to GDEPT and/or cytokine gene therapy as well as the impact of these therapies on tumor growth at the treatment site and distant sites.

Formulated Delivery of Enzyme/Pro-Drug and Cytokine Gene Therapy to Promote Immune Reduction of Treated and Remote Tumors in Mouse Models of Prostate Cancer

Principal Investigator: Pamela J Russell, PhD

Public Abstract

Prostate cancer is now the most common cancer and the second highest cause of cancer death in men in Western society. While early stage disease can be treated in several ways, late stage disease is presently incurable and pain management is often the only care that can be offered.

Gene therapies offer new possibilities for treatment, although these are still experimental. We are investigating the use of a gene therapy strategy, alone and in combination with hormones that can stimulate the immune system. The concept is that delivering a cell-killing agent to an accessible tumor, coupled with help from the immune system can promote a reduction in tumor size both at the treatment site and at remote tumor locations. In such gene therapy, a gene (in this case **a fusion gene of cytosine deaminase and uracil phosphoribosyltransferase (CD/UPRT) is used**) is delivered to a cancer cell so that harmless bacterial proteins are made. A second component, the pro-drug (in this case, **5 fluorocytosine (5-FC)**), is then given. Cancer **cells that make CD/UPRT convert 5-FC to a toxic compound** that kills the original cell and others nearby. Relatively little work has been done using this system, but it appears to be well suited to prostate cancer treatment because it has the capacity to kill slow growing tumors, which prostate cancers often are.

It is also thought that killing the tumor cells will attract other cells that form part of the immune system. We will identify and characterize these and will use hormones, known as cytokines to attract more of them into tumors. We will deliver the gene for the cytokine by itself and together with the gene because in other cancer studies, combination therapy works better.

A key factor in our work is how efficiently the therapeutic genes can be delivered to cancer cells. Certain viruses, especially human adenoviruses are commonly used for gene delivery because they get into cells efficiently. However, humans develop immunity to them as a result of natural infection. To improve delivery, we will use a **lentivirus** to deliver our cell-killing gene. Secondly, other studies have reported reagents that enhance the uptake of naked DNA, or plasmids. We will deliver the cytokine gene as a plasmid using a cationic lipid because this will allow the dose of cytokine to be adjusted independently of the cell-killing gene by varying the plasmid/virus ratio. Because cancers in the clinic are variable, formulating the reagent mix prior to injection should improve the chances that gene delivery to more tumor types will be efficient.

We will study these genes and reagents in two mouse models in which mouse prostate cancer cells grown in the laboratory are grown in the mouse prostate and in other sites in the body. The gene medicine reagents are then formulated and injected directly into prostate tumors, either separately, or in combination. We will monitor the number and type of immune cells that migrate into the tumor in response to gene-directed enzyme prodrug cell-killing or cytokine gene therapy as well as the impact of these therapies on tumor growth at the treatment site (the prostate) and at distant sites representative of the spread of the disease to other organs. If we are successful in reducing local and remote tumors in these model systems the work will provide valuable preclinical data to support a future clinical trial in humans.

STATEMENT OF WORK

Task 1. **GDEPT alone**: Assess the ability of delivery of a lentivirus expressing GDEPT (based on the fusion gene, cytosine daminase/uracil phosphoribosyltransferase (**CD/UPRT**) to suppress orthotopic and metastatic prostate cancer in RM-1 model. (Months 1-12).

a. Prepare recombinant lentivirus (using vector from Invitrogen).
b. Establish conditions for implanting TRAMP-C1 cells sc in TRAMP mice.
c. Optimize dose of virus needed to establish CD/UPRT GDEPT in orthotopically implanted RM-1 tumors when formulated with lipid and control plasmid.
d. Assess ability of optimal doses of CD/UPRT-GDEPT (and control plasmid) injected intraprostatically into RM-1 tumors together with systemic pro-drug (**5 fluorocytosine, 5FC**) treatment to suppress local prostate and metastatic (lung) tumor development.
e. Examine other tissues for signs of toxicity that might result from escape of the CD/UPRT GDEPT virus from the site of injection.
f. Identify by immunohistochemistry, the immune cell types infiltrating the tumors.

Task 2. **pCytokine work**: Assess the ability of lipid-based delivery of an , IL-12 or **IL-18** expressing plasmid (pCytokine) or a **combination of both** to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 12-22)

a. Prepare pCytosine constructs.
b. Determine dose of pCytosine-construct plasmid DNA (0.1-1.0 μg) which, when formulated with lipid and control virus leads to detectable expression of cytokine in orthotopically implanted RM-1 tumors by:
   (i) Harvesting tumor cells, culturing and monitoring cytokine production by Western blot.
   (ii) Measuring biological activity of secreted cytokine using a cytotoxic lymphocyte (CTL) bioassay.
   (iii) Measuring cytokine mRNA production by RT-PCR.
   (iv) Identify using immunohistochemistry the immune cell types infiltrating cytokine secreting tumors
c. Determine impact of administering 5FC prodrug on immune cell recruitment into tumors following injection of cytokine gene plasmid/lipid/control virus administration
d. Determine the persistence of cytokine production by transfected tumors.
e. Compare ability of transfected pCytokines (optimal dose complexed with lipid and control virus) to suppress orthotopic and metastatic RM-1 prostate tumor growth.
f. Choose optimal cytokine gene system on basis of maximum suppression of tumor growth obtained.

Task 3. **Combination therapy**: Assess the ability of delivery of a combined virus borne GDEPT and lipid delivered plasmid-borne cytokine gene therapy to suppress orthotopic and metastatic prostate cancer in RM-1 model and in TRAMP mice carrying sc TRAMP-C1 grafts. (Months 22-33)

a. Determine whether pCytokine-enhanced immune activity affects GDEPT.
b. Determine the effects of injecting lenti virus expressed GDEPT and pCytokine intraprostatically (using optimal doses of each component as revealed by Tasks 1A and 1B) on orthotopic tumor growth and metastases.

Task 4. **Tissue slice work**: Assess the ability of OAV encoding green fluorescence protein (GFP) under a prostate directed promoter from Dr Paul Rennie, Vancouver to express GFP in human tissue slices. (Months 24-33)

Task 5. Collate data, prepare reports and manuscripts (Months 33-36)

Reasons for our selections:
Task 1: Why CD/UPRT as suicide gene(s)?

Because of industrial constraints, we are no longer permitted to use purine nucleoside phosphorylase (PNP) from *E coli* as the "suicide" gene in GDEPT.
Based on the characteristics shown, we have selected the fusion gene, comprising cytosine deaminase and uracil phosphoribosyltransferase (CD/UPRT) also from *E coli* that has similar properties to PNP.

Advantage for use compared with other GDEPT systems	PNP	CD/UPRT
1. Conversion of prodrug to a metabolite that inhibits DNA and RNA synthesis, thereby making it suitable for use against dividing and non-dividing cells	PNP converts fludarabine phosphate to 2- fluoro-adenine that inhibits both DNA and RNA synthesis, making it suitable for use against dividing and non-dividing cells.	CD converts 5 fluorocytosine to 5 fluorouracil, whose metabolites, 5-fluoro-2'-deoxyuridine 5'monophosphate (5-FdUMP) and 5-fluorouridine 5'-triphosphate (5FUTP) damage DNA and RNA respectively. The rate-limiting step in the generation of 5-FdUMP and 5-FUTP is the formation of an intermediary metabolite, 5-fluorouridine mono-phosphate (5-FUMP). 5-FUMP is only be produced after a series of catalysed enzymatic reactions. This can be circumvented by the ability of UPRT to convert 5-FU directly to 5-FUMP thereby leading to more efficient production of anti-tumor metabolites, 5-FdUMP and 5-FUTP (Tiraby *et al.*, 1998). Thus drugs generated by CD/UPRT can kill both dividing and non-dividing cells.
2. Toxic metabolite is a small molecule that can freely diffuse between and into cells to establish a large bystander effect.	2 fluoroadenine (2FA) can readily diffuse and is associated with a large bystander effect killing infected and nearby non-infected cells (Sorscher *et al.*, 1994).	Metabolites of 5 fluorocytosine can produce a local bystander effect (see references below).
3. GDEPT generates a "distant bystander" effect.	PNP-GDEPT has been shown in our preliminary studies to generate a "distant bystander" effect , the mechanism for which requires further investigation.	CD-GDEPT has been shown to generate a distant bystander effect against colon carcinoma of the liver that was largely mediated by natural killer cells (Pierrefite-Carle *et al.*, 1999).

Preclinical studies using CD have reported its activity as a "suicide" gene in GDEPT for several cancers including prostate cancer (Anello *et al.*, 2000; Uchida *et al.*, 2001; Yoshimura *et al.*, 2002), colorectal (Pierrefite-Carle *et al.*, 1999), hepatocellular (Kanai *et al.*, 1997) and glioma (Adachi *et al.*, 2000), and a strong local bystander effect was observed. Moreover, CD-GDEPT has been safely trialed in Phase I studies either alone for colon carcimona of the liver (Crystal, 1997) or in combination with the Herpes Simplex virus thymidine kinase gene and ganciclovir GDEPT (TK-GDEPT) for local recurrent prostate cancer (Freytag *et al.*, 2002). UPRT sensitises cancer cells to low doses of 5-FU (Kanai *et al.*, 1998), and when used in conjunction with CD and 5-FC in GDEPT, was more effective than CD-GDEPT alone against colon cancer (Koyama *et al.*, 2000; Chung-Faye *et al.*, 2001) and glioma (Adachi *et al.*, 2000) *in vitro* and *in vivo*. There are no reports of this combination (CD/UPRT) being used agains prostate cancer, making this application novel.

Task 1: Why lentivirus?

It would be feasible to deliver the GDEPT using a modified human adenovirus, and such reagents are available commercially. However, most patients have been exposed to human adenoviruses and as a consequence, have generated antibodies that can neutralize human adenovirus vectors. Moreover, the immune system eliminates transduced cells that express viral proteins as well as the transgene, and therefore only transient transgene expression is observed. For this reason, we had chosen to use an ovine atadenovirus that had been shown not to be neutralized by human sera that neutralized human adenoviruses. However, for industrial reasons, we are not permitted to continue work with this virus.

Recently an adverse effect has been described in a infant with severe combined immunodeficienty (SCID) who was treated with cells transduced with a Moloney murine leukemia viral vector (a retrovirus vector) expressing a normal copy of the common γ-chain (γc) of the lymphocyte receptors for interleukin-2 and many other cytokines. Through random insertion of the virus into the child's genome, near a gene LM02 that is involved in angiogenesis, monoclonal T cell-leukemia developed in the child by a process of insertional mutagenesis (Verma editorial, 2002). Insertional mutagenesis appears to be a very rare event.

We have chosen to use a **lentivirus** (vector available from Invitrogen) for delivery of GDEPT. Lentiviruses, unlike other retroviruses, are able to transduce nondividing cells (Pfeifer *et al.*, 2001). This is important in prostate cancer, where the percentage of dividing cells is low (Berges *et al.* 1995). The HIV-1 preintegration complex has karyophilic properties due to the presence of nuclear localization signals (NLS) in the viral proteins matrix and integrase. These unique features allow the preintegration complex to cross the nuclear membrane using the cellular nuclear import machinery in the absence of mitosis (reviewed in Kootstra and Verma, 2003). Moreover, the duration of expression of the transgene is long, and the lentivirus is not affected by pre-existing immunity (except in HIV-1 patients) (Kootstra and Verma, 2003). Any transgene expressing cells should be killed by the administration of prodrug that will be converted to 5FU and its products, and hence should not pose a safety problem. Biodistribution and toxicity studies of an HIV-1 based vector, HR'cmvGFP, have shown that high levels of transgene are expressed in the liver, spleen and bone marrow 4 days after intravenous injection of BALB/c mice, but by 40 days after injection, only bone marrow exhibited a consistently high level of transgene (Pan *et al.*, 2002). Between 0 and 1% of transgene was detected in all other organs. No significant pathologic lesions were found attributable to vector in any of the tissues examined.

Task 2: Why IL-12, IL-18 and a combination?

When we wrote the original grant, we wished to use IL-3, IL-12 or IL-15 for the reasons stated in the grant application. Recently, it has been shown that there is marked synergy between IL12 and IL-18, making this a worthwhile combination to test. Limitations of time will not permit us to test all of the different cytokines, and there have been some reports of immune enhancement using other GDEPT systems plus IL-12 (Hall *et al.*, 2002), making this a worthwhile candidate. For this reason, we have decided to concentrate on using IL-12 and IL-18 alone or in combination. IL-18 has been shown to share some of its biological functions with IL-12, including the induction of IFN-γ production, the enhancement of natural killer (NK) cell cytotoxicity and the enhancement of activated T cell proliferation (Trinchieri *et al.*, 1995a, b). In addition, IL-18 upregulates the expression of the cell-killing molecule, FasL on the surface of NK cells involved in the T helper 1 response (Th1) (Tsutsui *et al.*, 1996). IL-18 deficient mice displayed reduced production of IFN-γ, impaired NK cell activity and defective Th1 cell responses (Takeda *et al.*, 1998). Mice deficient in IL-18 and IL-12 showed further diminished NK cell activity and Th1 cell responses (Takeda *et al.*, 1998). Robinson *et al* (1997) have shown that IL-18 as well as IL-12 show marked synergy in inducing IFN-γ production from differentiating Th1 cells, suggesting that both cytokines are required for significnt expression of the Th1 phenotype. Furthermore, IL-12 can upregulate the production of the IL-18 receptor α (IL18Rα) chain on Th1 cells (Ahn *et al.*, 1997) whereas IL-18 has been shown to upregulate the IL12Rβ2 chain on Th1 cells (Chang *et al.*, 2000). This shared upregulation of receptors provides a positive feedback mechanism allowing these cytokines to act synergistically. Anti-tumor efficacy by IL-18 has recently been shown inother cancer models including melanoma (Nagai *et al.*, 2002), breast cancer (Nakata *et al.*, 1999) and in combination with IL-12 in renal carcinoma (Hara *et al.*, 2001).

Task 4: Prostate specific promoter for use in tissue slice work:

Due to industrial constraints, we are no longer permitted to use the prostate specific promoter that we have used in our previous studies. We are currently negotiating with Dr Paul Rennie and his team from The Prostate Center at Vancouver General Hospital, Canada, for collaborative studies using a lentiviral vector that expresses the enhanced green fluorescent protein reporter (EGFP) gene under a probasin promoter: (-426/+28) dimer known as ARR_2PB. These lentivirus constructs have been shown to express EGFP in a prostate-directed and androgen-regulated manner in human prostate cancer cells in vitro (Duan *et al.*, 2002).

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